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RESEARCH PAPER

The products of the broken *Tm-2* and the durable *Tm-2²* resistance genes from tomato differ in four amino acids

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Abstract

To gain an insight into the processes underlying disease resistance and its durability, the durable *Tm-2²* resistance gene was compared with the broken *Tm-2* resistance gene. The *Tm-2* gene of tomato could be isolated via PCR with primers based on the *Tm-2²* sequence. The *Tm-2* gene, like the *Tm-2²* gene, encodes an 861 amino acid polypeptide, which belongs to the coiled coil/nucleotide binding site/leucine-rich repeat class of resistance proteins. The functionality and the nature of the isolated *Tm*-2 gene were confirmed by introducing the gene under the control of the 35S promoter into tomato mosaic virus-susceptible tobacco. This transgenic tobacco was crossed with transgenic tobacco plants producing the movement protein (MP)-authenticated MP as the Avr protein of the *Tm-2* resistance. The *Tm-2²* and *Tm-2* open reading frames only differ in seven nucleotides, which on a protein level results in four amino acid differences, of which two are located in the nucleotide binding site and two are located in the leucine-rich repeat domain. The small difference between the two proteins suggests a highly similar interaction of these proteins with the MP, which has major implications for the concept of durability. Comparison of the two resistance-conferring alleles (*Tm-2* and *Tm-2²*) with two susceptible alleles (*tm-2* and *lptm-2*) allowed discussion of the structure–function relationship in the Tm-2 proteins. It is proposed that the Tm-2 proteins display a partitioning of the leucine-rich repeat domain, in which the N-terminal and C-terminal parts function in signal transduction and MP recognition, respectively.

Key words: *Lycopersicon esculentum*, *Lycopersicon peruvianum*, *Tm-2*, *Tm-2²*, tomato mosaic virus, plant disease resistance gene, durability, structure–function relationships.

Introduction

Due to breeding and selection for economically valuable traits, crops usually have little variation in their gene pool. Consequently, their resistance to harmful changes or events, like the appearance of a new pathogen, and their adaptability to changing demands are limited. This limited genetic diversity can lead to devastating disasters, which is exemplified by the Irish Potato Famine of the 1840s. Resistance against pathogens requires the presence of resistance (R) genes, whose polypeptide products recognize products of the pathogen and, subsequently, are able to trigger a defence response. These R genes could have been lost from the gene pool due to breeding or could have been absent in the original founder material, which presents a problem to breeders. A commonly used method to increase the genetic flexibility of commercial crops is to make use of the gene pool of closely related wild relatives of the crops. In this way, valuable new genes can be introduced into crops by introgression. Well-studied and successful examples of this procedure for R genes are the introductions of the *N*-gene of *Nicotiana glutinosa*, conferring resistance against tobacco mosaic virus (TMV), into *N. tabacum* (Dinesh-Kumar *et al.*, 1995; Marathe *et al.*,

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Abbreviations: Avr, avirulence; CaMV, cauliflower mosaic virus; CAPS, cleaved amplified polymorphic sequence; CC, coiled coil; CNL, coiled coil/nucleotide binding site/leucine-rich repeat; LRR, leucine-rich repeat; MP, movement protein; NB-ARC domain, nucleotide binding site–apoptosis, R gene products, and CED-4 domain; ORF, open reading frame; R, resistance; SCAR, sequence characterized amplified region; TMV, tobacco mosaic virus; ToMV, tomato mosaic virus.

2002), and the *Cf*-genes of *Lycopersicon pimpinellifolium*, conferring resistance against the fungus *Cladosporium fulvum*, into *L. esculentum* (Boukema, 1980). Another example of this procedure is the introgression of tomato mosaic virus (ToMV) resistances into the cultivated *L. esculentum*. ToMV infections in modern commercial *L. esculentum* varieties are controlled by the *Tm-1*, *Tm-2*, and *Tm-2*^{2} R genes (Pelham, 1966; Hall, 1980), which were introgressed from the wild tomato species *L. hirsutum* (*Tm-1*) and *L. peruvianum* (*Tm-2* and *Tm-2*^{2}).

Genetic analysis of ToMV strains capable of overcoming the resistances has shown that for *Tm*-1 the *RNA-dependent RNA polymerase* gene of ToMV is the matching *Avirulence* (*Avr*) gene (Meshi *et al.*, 1988). The *Tm-2* and the *Tm-2*^{2} resistances are considered to be allelic (Khush *et al.*, 1964; Pelham, 1966; Schroeder *et al.*, 1967; Hall, 1980; Tanksley *et al.*, 1992) and share the movement protein (MP) of ToMV as the matching Avr protein. The *Tm-2*^{2} gene was recently isolated from tomato and demonstrated to be functional in both tomato and tobacco (Lanfermeijer *et al.*, 2003, 2004). The Tm-2^2 protein displays all the characteristics of the coiled coil/nucleotide binding site/leucine-rich repeat (CNL) type of R proteins and differs considerably from the polypeptide encoded by the allele which was isolated from susceptible *L. esculentum* lines (*tm-2*). The differences are concentrated in the C-terminal half of the leucine-rich repeat (LRR) domain.

The *Tm-2* and *Tm-2*^{2} resistances have characteristics which make the study of the *Tm-2*^{2} locus meaningful for the study of structure–function relationships of the CNL-type of R proteins. Firstly, the two resistances share the viral MP as the Avr but, in order to break the two resistances, mutations at different locations in the MP are necessary (Meshi *et al.*, 1989; Calder and Palukaitis, 1992; Weber *et al.*, 1993; Weber and Pfitzner, 1998). Secondly, the *Tm-2*^{2} resistance has been more durable than the *Tm-2* resistance (Fraser *et al.*, 1989). Consequently, the *Tm-2*^{2} resistance is still used in tomato breeding and, therefore, is of ongoing practical and economical importance. In particular, the combination of the three aspects—the sharing of MP as the Avr protein, the different locations of the mutations necessary for circumventing the resistances, and the different durabilities—is intriguing.

Here, the isolation and characterization of the resistance-conferring allele *Tm-2* of the *L. esculentum* variety GCR236 (originally introgressed from *L. peruvianum*) and the *lptm-2* allele of a ToMV-susceptible *L. peruvianum* are reported. The genes were obtained by PCR using primers developed on the basis of the sequence of the *Tm-2*^{2} gene. With the differences between the two resistance-conferring alleles as a starting point, possible reasons for the differences between the nature of the *Tm-2*^{2} and *Tm-2* genes is discussed.

Materials and methods

Isolation of the Tm-2 *allele from* L. esculentum*, accession Craigella GCR236 and the* lptm-2 *allele from* L. peruvianum *(CGN14355)*

The *Tm-2*^{2} locus contains only a single gene (Lanfermeijer *et al.*, 2003), which simplifies the cloning of the homologous *Tm-2* and *lptm-2* alleles using the *Tm-2*^{2}-specific primer set PrRuG84/PrRuG86 (Table 1). PCR was performed on genomic DNA using Platinum Taq (Life Technologies) or ExTaq (TaKaRa Bio Inc). Three independent PCR products for each primer set were cloned into pGEM-T-Easy Vector (Promega) and their nucleotide sequences determined.

The introduction of the Tm-2 *open reading frame (ORF) in* Nicotiana tabacum *SR1*

The binary vector pTM90 was constructed with the *Tm-2* ORF under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the *NOS*-polyadenylation signal for the transformation of the *Nicotiana tabacum* SR1 line, which is susceptible to ToMV and TMV infections. Using primer PrRuG97 and PrRuG102 (Table 2), a PCR product containing the complete ORF of *Tm-2* with an introduced *Nco*I site at the ATG and an introduced *Nco*I site 11 bp downstream of the TGA, was amplified from genomic DNA of tomato line Craigella GCR236. The PCR product was digested with *Nco*I and this fragment was introduced into the *Nco*I site of pTM6 (Lanfermeijer *et al.*, 2003). The orientation of the ORF relative to the promoter and polyadenylation signal was assessed by digestion and the plasmid was named pMP1039. The pMP1039 vector was digested with *Asc*I and *Pac*I and the chimeric *Tm-2* gene was cloned into the binary vector pVictorHiNK, resulting in plasmid pTM90.

Table 1. *PCR-primers used in this study and their target sequences*

Underlined residues indicate introduced mutations in order to generate an *Nco*I restriction site. Primer sequences are given from 5′ to 3′.

Target	Name	Primer	Direction
Tm-2, *lptm-2*	PrRuG084	CTTGACAAGACTGCAGCGAGTGATTGTC	F
	PrRuG086	CTACTACACTCACGTTGCTGTGATGCAC	R
	PrRuG097	TTTTCCATGGCTGAAATTCTTCTTACATCAGTAATCAATAAATCTG	F
	PrRuG102	CTGACCTGCCATGGTGTTCATTTACTCAGCTTTTTAAGCC	R
	PrRuG151	GAGTTCTTCCGTTCAAATCCTAAGCTTGAGAAG	F
SCAR[a]	PrRuG248	AGCGTCACTCCATACTTGGAATAA	
	PrRuG249	AGCGTCACTCAAAATGTACCCAAA	
pTM90	PrRuG531	ACACGCTTGTCTACTCCAAA	F
	PrRuG532	GCGTTGTCAACATAAGATCG	R

[a] Primer sequences derived from Sobir *et al.* (2000).

Table 2. *Virus specificity of tomato and transgenic tobacco lines assessed by inoculation with various tobamovirus isolates*

Tomato accession	Genotype or introduced T-DNA	Tobamovirus isolates[a]					
		TMV	Cg	0	1	2	2A
GCR26 (Craigella)	*tm-2*, *tm-2*	+[b]	+	+	+	+	+
GCR236 (Craigella)	*Tm-2*, *Tm-2*	−	−	−	−	+	−
GCR267 (Craigella)	*Tm-2*^{2}, *Tm-2*^{2}	−	−	−	−	−	+
L. peruvianum (CGN14355)	*lptm-2*, *lptm-2*	n.d.[c]	n.d.	n.d.	+	+	+
Tobacco SR1	None	+	+	+	+	+	+
Tobacco SR1[d]	pTM90 (*Tm-2*)	−	−	−	−	+	−

[a] Virus isolates: TMV, tobacco mosaic virus-U1 isolate; Cg, TMV-Cg isolate (an *Arabidopsis*- and tobacco-infecting tobamovirus); 0, ToMV-GdK (wild-type tomato mosaic virus); 1, ToMV-SPS (*Tm-1* breaking isolate); 2, ToMV-GeRo (*Tm-2* breaking isolate); 2A, ToMV-GM65 (*Tm-2*^{2} breaking isolate). Virus isolates were obtained from Plant Research International, Wageningen, The Netherlands, except TMV-Cg which was obtained from Dr Masayuki of Hokkaido University, Japan.

[b] + indicates infection, − indicates no infection.

[c] Not determined.

[d] Five independent lines (F0071, F0076, F0078, F0080, and F0085) were tested and all displayed the same virus specificity.

The plasmid pTM90 was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Transformants were selected on L-Broth plates. Subsequently, the transformants were checked for unaltered gene constructs and used to transform *Nicotiana tabacum* SR1 leaf explants as described (Horsch *et al.*, 1985; Lanfermeijer *et al.*, 2004). After culturing the explants on the appropriate media in order to stimulate shoot and root development, kanamycin-resistant plantlets were transferred to soil and grown in the greenhouse under standard greenhouse conditions.

Seeds, obtained from self-pollination or crosses, were sown and grown on Murashige–Skoog plates, supplemented with 1% w/v sucrose and, if necessary, 100 μg m^{-1} kanamycin and, subsequently, transferred to soil and grown in the greenhouse under standard greenhouse conditions.

Transgenic plants with the *Tm-2* gene were selected on the basis of two criteria: the ability to grow in the presence of kanamycin, and the presence of the T-DNA. The presence of the T-DNA was assessed using PCR with the T-DNA-specific primers, PrRuG531 and PrRuG532 (Table 1). DNA that served as a template for these assays was isolated from the tobacco plants according to the alkali treatment (Klimyuk *et al.*, 1993).

Virus resistance assays

Lycopersicon species and *N. tabacum* plants to be tested were infected with leaf homogenates of *N. tabacum* plants infected with Dutch greenhouse isolates of ToMV (Lanfermeijer *et al.*, 2003). In experiments with transgenic plants, untransformed plants were used as controls for virus inoculations. The plants were all inoculated twice with a 4 d interval to rule out random escape of inoculation. Virus symptoms were visually monitored on a daily basis for the duration of the experiment (21 d). After 21 d a leaf homogenate of the infected plants was inoculated onto the ToMV-indicator plant *N. glutinosa*, and lesions were scored after 3–4 d. Infection of *N. glutinosa* with TMV and ToMV-containing solutions resulted in the development of local lesions on the infected leaves.

CAPS and SCAR markers for discrimination of the tm-2*,* Tm-2*, and* Tm-2^2 *alleles*

PCR was performed on template-material obtained through the alkali treatment (Klimyuk *et al.*, 1993). Approximately 1 mm^2 of treated leaf-tissue was transferred to 25 μl of PCR solution. This solution consisted of 0.625 U of Taq DNA polymerase (Roche Diagnostics) in its prescribed reaction buffer with either 5 μM of the primers PrRuG086 and PrRuG151 [CAPS (cleaved amplified polymorphic sequence) markers] or 5 μM of the primers PrRuG248 and PrRuG249 [SCAR (sequence characterized amplified region) markers] (Sobir *et al.*, 2000) (Table 1). The PCR protocol used for both marker-types was: 5 min at 94 °C, followed by 30 cycles that consisted of 15 s at 94 °C, 45 s at 55 °C, and 90 s at 72 °C. The protocol was concluded with 5 min at 72 °C. Fifteen microlitres of the PCR products were subsequently digested with *Hpa*I, *Bfr*I, or *Acc*I.

Analysis software

Sequences were analysed using the ClustalW (Thompson *et al.*, 1994), the Clone Manager Software (Scientific and Educational Software), and Blast (Altschul *et al.*, 1990) software.

Accession numbers

The accession numbers for the *Lycopersicon esculentum Tm-2*^{2}, *Tm-2*, and *tm-2* genes and the *Lycopersicon peruvianum lptm-2*^{2} gene are AF536201, AF536200, AF536199, and AY765395, respectively.

Results

Molecular cloning of the Tm-2 *gene from* L. esculentum

Isolation of the *Tm-2* allele necessitates assessment of the genotypes and phenotypes of the tomato accessions used from which it was derived. The Craigella accessions, which contain either the *Tm-2* (Craigella GCR236) or the *Tm-2*^{2} allele (Craigella GCR267), could only be infected with their breaking virus isolates, ToMV-2 or ToMV-2A, respectively. The susceptible Craigella (Craigella GCR26), which contains the *tm-2* allele (Lanfermeijer *et al.*, 2003), and the *L. peruvianum* plants could be infected with all the isolates used (Table 2). Additionally, the genotype of the Craigella accessions was confirmed using SCAR markers (Sobir *et al.*, 2000) and new CAPS markers developed from the known sequences of *tm-2* and *Tm-2*^{2} and the sequence of *Tm-2* presented here. These CAPS markers were based on the absence of an *Hpa*I site in *tm-2*, which is present in both *Tm-2* and *Tm-2*^{2}, and the sole presence of a *Bfr*I site in *Tm-2*^{2}. The PCR product, obtained with the use of the primers PrRuG086 and PrRuG151, was either treated with

*Hpa*I or *Bfr*I. By the combination of the results of the two CAPS markers the genotype of a plant can unambiguously be determined (Fig. 1). Tomato accessions (Craigella GCR26 and ATV840) which contain the *tm-2* allele (Lanfermeijer *et al.*, 2003) did not display digestion of the PCR product by both *Hpa*I and *Bfr*I, whereas for the *Tm-2*-carrying accession (Craigella GCR236) only *Hpa*I digested the PCR product. The PCR product obtained from *Tm-2*^{2}-containing accessions (Craigella GCR267 and ATV847; Lanfermeijer *et al.*, 2003) was digested by both enzymes.

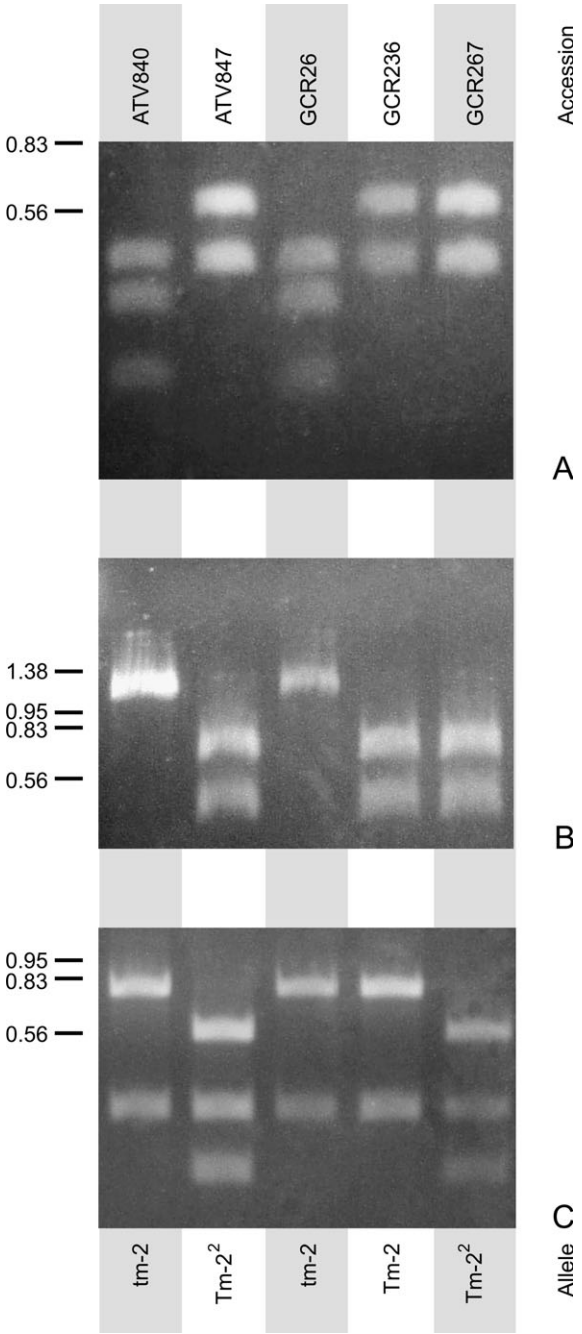

Fig. 1. The genotypes of various tomato accessions. The genotypes of five tomato accessions, either used in this study or in a previous study (Lanfermeijer *et al.*, 2003), were determined by SCAR and CAPS markers. (A) SCAR markers for discriminating between *tm-2*, on the one hand, and *Tm-2* and *Tm-2*^{2}, on the other hand. PCR and restriction were performed according to Sobir *et al.* (2000). (B, C) CAPS markers for the discrimination between *tm-2*, *Tm-2*, and *Tm-2*^{2}: (B) *Hpa*I-dependent marker; (C) *Bfr*I-dependent marker. See Results for an explanation of the CAPS markers.

Lycopersicon esculentum contains a single *Tm-2*^{2}-like gene (Lanfermeijer *et al.*, 2003), which allows the cloning of the homologous *Tm-2* allele using the *Tm-2*^{2}-specific primer set PrRuG84/PrRuG86 (Table 1). A 2875 bp PCR product was obtained, which was identical in size to similar PCR products previously obtained from the *tm-2* and *Tm-2*^{2} alleles (Lanfermeijer *et al.*, 2003). Analysis and comparison of the sequence of the PCR product of *Tm-2* which was obtained revealed that it contained an intact ORF of a size identical to that of *Tm-2*^{2}. This ORF could be translated into a polypeptide 861 amino acids long, which resembled the Tm-2^2 protein and, consequently, contained all the features of the CNL class of R proteins (Hammond-Kosack and Jones, 1997; Jones and Jones, 1997; van der Biezen and Jones, 1998; Lanfermeijer *et al.*, 2003). Alignment of the *Tm-2* and *Tm-2*^{2} ORFs and their polypeptides revealed that the difference between the *Tm-2* allele and *Tm-2*^{2} was unexpectedly small; on the DNA level, seven differences (0.3%) were observed which result, on the protein level, in four differences (0.5%) (Fig. 2).

Introduction of the Tm-2 *ORF in* Nicotiana tabacum *SR1*

Final confirmation of the isolation of the tobamovirus R gene came from the transformation of the *Nicotiana tabacum* SR1 line, which is susceptible to ToMV and TMV infections, with the *Tm-2* gene under the control of the CaMV 35S promoter and the *NOS*-polyadenylation signal. Primary kanamycin-resistant transformants were grown in the greenhouse and three cuttings were taken from each individual plant. The cuttings were inoculated with a Dutch wt-ToMV isolate (ToMV-GdK; Table 2). All cuttings of all primary transformants were resistant towards infection with ToMV. Also, as with the *Tm-2*^{2} gene in tomato (Lanfermeijer *et al.*, 2003) and in transgenic tobacco (Lanfermeijer *et al.*, 2004), no macroscopically visible symptoms, like local lesions, were observed in tobacco plants containing the *Tm-2* R gene. Control tobacco plants displayed the characteristic mosaic symptoms of infection. Inoculation of leaves of the ToMV-indicator species, *N. glutinosa*, with leaf sap from the mosaic-displaying plants resulted in the development of local lesions, whereas these leaves, when inoculated with leaf sap from the symptom-free transgenic plants, developed no local lesions. All five transformants analysed contained the T-DNA as detected by PCR with primers pRUG531 and pRUG532 (Table 1; data not shown). In the progeny of the primary transformants similar results were obtained: all kanamycin-resistant plants contained

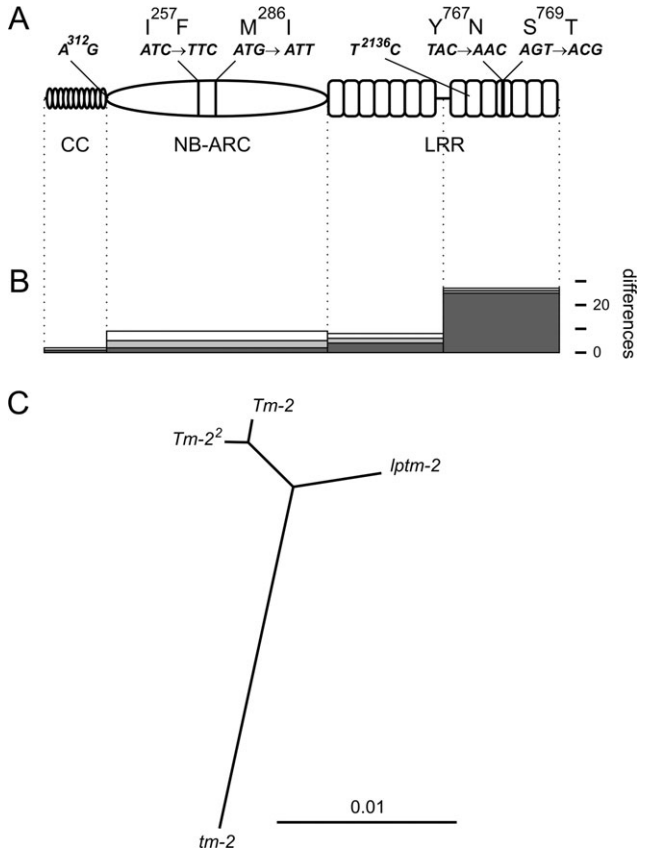

Fig. 2. Map of the differences between the genes and the polypeptides of *Tm-2* and *Tm-2*^{2}. (A) A schematic representation of the R protein with the differences between *Tm-2* and *Tm-2*^{2}. Small elipses indicate leucine zipper motifs, the large elipse represents the NB-ARC domain, and the oblongs represent LRRs. Symbols identified in roman font: amino acid differences (Tm-2^2 versus Tm-2) with, in superscript, their location; symbols identified in italic font: nucleotide differences (*Tm-2*^{2} versus *Tm-2*) with, in superscript, their location. Where a nucleotide change results in an amino acid change, the complete codon, of which the nucleotide is a part, and the amino acids are shown with, in superscript, the location of the amino acid. (B) Histogram displaying the number of amino acid differences per domain between the *tm-2* allele and three *peruvianum* alleles (*lptm-2*, *Tm-2*, and *Tm-2*^{2}). White area: number of locations at which the tm-2 protein differs from only one *peruvianum* protein; light grey area: number of locations at which the tm-2 protein differs from two *peruvianum* proteins; dark grey area: number of locations at which the tm-2 protein differs with all three *peruvianum* proteins. (C) Unrooted tree of the alleles *lptm-2*, *tm-2 Tm-2*, and *Tm-2*^{2}. The boot-strapped tree was generated using the program Clustal X. The bar indicates 0.01 residue change per residue.

the T-DNA as detected by PCR and were resistant to wt-ToMV. Moreover, kanamycin resistance of the progeny from self-pollinated plants displayed a Mendelian 'three to one' segregation, indicative of the presence of a single transgene in the primary transformants.

Virus-specificity of the N. tabacum *SR1 expressing the* Tm-2 *transgene*

Homozygous F_2 tobacco plants from five independent transgenic lines expressing the *Tm-2* gene were analysed for their virus specificity. For these experiments six tobamoviruses were used: TMV; ToMV-GdK (wild-type tomato mosaic virus); Isolate 1, ToMV-SPS (*Tm-1* breaking isolate); Isolate 2, ToMV-GeRo (*Tm-2* breaking isolate); Isolate 2A, ToMV-GM65 (*Tm-2*^{2} breaking isolate); and TMV-Cg (an *Arabidopsis*- and tobacco-infecting tobamovirus). The virus specificity of the transgenic *Tm-2* gene in the tobacco background was similar to the virus specificity of the tomato-accession Craigella GCR236, which was the source of the gene. Both the GCR236 and the transgenic tobacco lines (F0071, F0076, F0078, F0080, and F0085) were resistant against TMV-U1, ToMV-GdK, ToMV-SPS, ToMV-GM65, and TMV-Cg, but could be infected by ToMV-GeRo, the *Tm-2*-breaking ToMV-isolate (Table 2). These observations demonstrate that next to the preservation of the ability of conferring resistance, the characteristics of the *Tm-2* R gene are also conserved after transformation of the *Tm-2* gene into a susceptible tobacco background, which confirms the isolation of the *Tm-2* gene. Moreover, as for the *Tm-2*^{2} gene, the use of the CaMV 35S promoter did not influence the characteristics of the *Tm-2* gene (Lanfermeijer *et al.*, 2004).

Crosses between tobacco plants, which express the ToMV-MP *gene, and tobacco plants with the* Tm-2 *transgene*

The *Tm-2*^{2} gene was isolated from tomato through transposon tagging and making use of the lethal combination of the presence of *MP* transgene and the *Tm-2*^{2} being expressed in the same plant (Weber and Pfitzner, 1998; Lanfermeijer *et al.*, 2003). The same combination was tested to assess the functioning of the *Tm-2* gene in tobacco. Homozygous MP-containing tobacco plants (Lanfermeijer *et al.*, 2004) were crossed with homozygous ToMV-resistant tobacco plants containing the *Tm-2* transgene. However, contrary to the observations made on the cross between MP-containing tobacco and *Tm-2*^{2}-containing tobacco (Lanfermeijer *et al.*, 2004), the progenies of plants containing the *MP* or the *Tm-2* transgenes obtained by self-pollination or by crossing the two genotypes, displayed all germination frequencies in the order of 80% (Table 3). However, seedlings from the cross between plants containing the *MP* and the *Tm-2* gene displayed a severe growth arrest. Roots and shoots from these seedlings did not develop properly. The cotyledons were present and the first true leaves were initiated but neither expanded even after 28 d (Fig. 3).

The lptm-2 *allele*

The *tm-2* allele is considered to have originated in *L. esculentum*, whereas the *Tm-2* and *Tm-2*^{2} alleles originated in *L. peruvianum* (Lanfermeijer *et al.*, 2003). In order to study the relationship between *tm-2*, *Tm-2*, and *Tm-2*^{2}, and to determine what role the origin of the alleles

Table 3. *Percentage of progeny of crosses between transgenic tobacco lines which are homozygous for the* Tm-2 *gene and one which is homozygous for the* ToMV-MP *gene that display the arrested growth phenotype (Fig. 3)*

Parental lines	Genotype	Germination frequency	Arrested growth phenotype (%)	No. of seeds	No. of crosses
MP (selfing)	(*MP*,*MP*;−,−)	77±7	0	532	5
MP×*Tm-2*	(*MP*,−; *Tm-2*,−)	79±10[a]	99±1[a]	879	9
Tm-2 (selfing)	(−,−; *Tm-2*,*Tm-2*)	89±9	0	845	5

[a] Average of nine crosses (all five independent transgenic lines were at least crossed once with MP-containing plants).

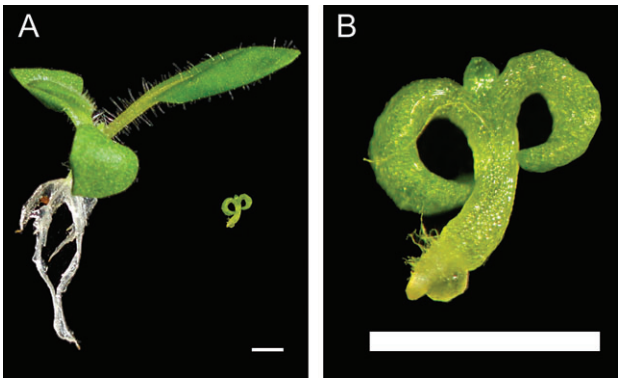

Fig. 3. Phenotype of seedlings from the cross between MP-containing and Tm-2-containing transgenic tobacco plants. In both panels the white bar represents 2 mm. (A) Left: a wild-type seedling 28 d after germination; right: a seedling from a seed that originates from a cross between an *MP*- and a *Tm-2*-containing transgenic plant, 28 d after germination. Both seedlings were grown *in vitro*. (B) An enlarged picture of the seedling that is shown in (A) and that originates from a cross between an *MP*- and a *Tm-2*-containing transgenic plant.

might play in their difference, a fourth *tm-2*-like gene was isolated from *L. peruvianum* var. *dentatum* accession CGN14335 by PCR using the *Tm-2*^{2}-specific primers PrRuG97 and 102 (Table 1). The 2875 bp PCR product obtained contained one single ORF that encoded a protein identical in size and make-up to the Tm-2^2 protein. Because the accession, from which this allele was obtained, was susceptible to all ToMV strains tested (Table 2), this allele was named *lptm-2*. The difference between *lptm-2* and *Tm-2*^{2} was less than the difference between *Tm-2*^{2} and *tm-2* (Lanfermeijer *et al.*, 2003). Twenty-six nucleotide differences (1.0%) were observed between *lptm-2* and *Tm-2*^{2} that resulted in 15 amino acid differences (1.7%). Calculating the phylogenetic relationship between the four alleles resulted in an unrooted tree as presented in Fig. 2C. A clear grouping of the alleles *Tm-2*^{2}, *Tm-2*, and *lptm-2* can be observed, which is in agreement with the fact that the *Tm-2* and *Tm-2*^{2} R genes were introgressed into *L. esculentum* from *L. peruvianum* (Pelham, 1966) and that *tm-2* is probably an original allele of *L. esculentum*.

Discussion

In this paper, the isolation of the *Tm-2* gene from *L. esculentum* is described. The *Tm-2* gene is the second allele of the *Tm-2*^{2} locus that confers resistance to tobamoviruses. Its ability to confer resistance to tobamoviruses was confirmed by the introduction of this gene into *N. tabacum* SR1. The *Tm-2* and *Tm-2*^{2} alleles have always been considered to be allelic (Khush *et al.*, 1964; Pelham, 1966; Schroeder *et al.*, 1967; Hall, 1980; Tanksley *et al.*, 1992), which is now confirmed by the sequence of the *Tm-2* allele. However, it had already been observed that the two resistance-conferring alleles, *Tm-2* and *Tm-2*^{2}, have different levels of durability (Hall, 1980; Fraser *et al.*, 1989). Whereas the *Tm-2*^{2} allele has been used for four decades, the *Tm-2*-conferred resistance was broken by ToMV soon after its introduction in commercial *L. esculentum* varieties. The present isolation of the *Tm-2* allele, together with the recent isolation of the *Tm-2*^{2} allele (Lanfermeijer *et al.*, 2003) allows comparison of these two alleles and the possibility of gaining insight in the reasons for their different characteristics (Table 4).

The molecular differences between the proteins encoded by the alleles tm-2, lptm-2, Tm-2, *and* Tm-2^2

The four proteins, encoded by the four alleles of the *Tm-2*^{2} locus, have an identical structure, with all the typical elements of the CNL type of R proteins (Hammond-Kosack and Jones, 1997; Jones and Jones, 1997; van der Biezen and Jones, 1998; Lanfermeijer *et al.*, 2003). The different characteristics of the Tm-2 and Tm-2^2 resistances should reside in their amino acid composition, but, unexpectedly, the difference between the Tm-2^2 and the Tm-2 protein is surprisingly small. Only four amino acid differences are present between the proteins of these two alleles (Fig. 2A). Of these four amino acid differences, two are located in the NB-ARC (nucleotide binding site–apoptosis, R gene products, and CED-4 domain) domain [Ile^{257}Phe and Met^{286}Ile (Tm-2^2 versus Tm-2)] whereas the other two are in the LRR domain (Tyr^{767}Asn and Ser^{769}Thr) (Fig. 2A). The differences at positions 257 and 286 are in the motifs III and IV of the NB-ARC domain (Pan *et al.*, 2000) but at these two positions both amino acids are allowed based on the functionality of the Tm-2 and Tm-2^2 proteins and the alignments of R genes from *Arabidopsis* and tomato (Pan *et al.*, 2000; Meyers *et al.*, 2003). Whether these two differences are involved in the difference between the virus specificity of two alleles has to be studied, because it has been observed that regions outside the LRR domain can affect the specificity of R proteins (Ellis *et al.*, 1999; Luck *et al.*, 2000). However, based on the predominant view that the LRR domain is the major determinant in R-protein specificity and that the NB-ARC domain functions in signal transduction (Jones and Jones, 1997; Bittner-Eddy *et al.*, 2000; Ellis *et al.*, 2000; Halterman *et al.*, 2001; Moffett

Table 4. *Differences between the* Tm-2 *and* Tm-2^2 *resistances*

Resistance	*Tm-2*	*Tm-2*^{2}
Amino acids	Phe257; Ile286; Asn767; Thr769	Ile257; Met286; Tyr767; Ser769
Characteristics of the breaker virus	Virulent	Crippled
Location of the mutations of the breaker virus	N-terminal and central regions of MP	C-terminus of MP
Phenotype of cross with MP-containing plants	Arrested growth	No germination

et al., 2002; Tameling *et al.*, 2002; Belkhadir *et al.*, 2004) it is most likely that the difference in virus specificity is caused by the amino acid differences at positions 767 and 769. This is supported by the location of these residues, namely in or close to the β-sheet of the LRR, which is thought to be the area involved in the protein–protein interactions (Jones and Jones, 1997).

The differences between the protein encoded by the *esculentum*-allele (*tm*-2) and the proteins encoded by the so-called *peruvianum* alleles (*lptm-2*, *Tm-2*, and *Tm-2*^{2}) concentrate in the C-terminal half of the LRR domain, as was already observed for the *tm-2* and *Tm-2*^{2} alleles (Fig. 2B; Lanfermeijer *et al.*, 2003). Recently, it has become clear that the LRR domain of CNL proteins can be separated into two subdomains: the N-terminal half, that fulfils a role in the signal transduction, and the C-terminal half, that fulfils a role in recognition of the Avr protein and its virulence target (Moffett *et al.*, 2002; Rathjen and Moffett, 2003; Belkhadir *et al.*, 2004). The higher degree of conservation in the N-terminal subdomain of the Tm-2^2-LRR domain is in accordance with a role of this subdomain in signal transduction. The interactions of this subdomain, either intramolecular or extramolecular with downstream signal transduction elements, will be conserved and, therefore, do not allow for much freedom for variation in the amino acid sequence. The larger variation in the C-terminal subdomain is a consequence of its role in the recognition of the Avr proteins, whether or not in complex with their virulence target (Jones and Jones, 1997; Ellis *et al.*, 2000; Glazebrook, 2001; Van der Hoorn *et al.*, 2002). This role necessitates flexibility in order to counteract the changes of Avr proteins, which are induced by a pathogen in order to circumvent the resistance.

Moreover, the eighth LRR (Lanfermeijer *et al.*, 2003) contains a large number of proline residues, which are structurally unfavourable for the formation of either α-helices or β-sheets and might hamper the formation of this eighth LRR. This might suggest that this region of the Tm-2 proteins does not adopt an LRR structure and, instead, forms a hinge or linkage domain between the two functionally separate LRR subdomains (Fig. 2A), comparable to the molecular hinge in the LRR domain of the Cf proteins (Hammond-Kosack and Jones, 1997).

The interaction between the two R proteins and MP

On the interaction between the Tm-2 and Tm-2^2 R proteins and MP, several, and at first sight contradictory, observations have been made. Firstly, the amino acid changes in MP that are necessary to overcome the *Tm-2* and *Tm-2*^{2} resistances are located in different parts of MP. For the *Tm-2* resistance those changes are located in the N-terminal half (Meshi *et al.*, 1989; Calder and Palukaitis, 1992), whereas those necessary to overcome the *Tm-2*^{2} resistance are located in the C-terminal half of MP (Weber and Pfitzner, 1998; Calder and Palukaitis, 1992; Weber *et al.*, 1993). This suggests that the two R proteins interact with different domains of MP. Secondly, the experiments performed by Weber *et al.* (2004) indicate that the interactions of the Tm-2 and Tm-2^2 proteins with MP are not that different. They showed that transgenic expression of a gene, which encodes a protein consisting of the first two-thirds of MP, in tomato plants with either the *Tm-2* or *Tm-2*^{2} resistance results in a hypersensitive response, while expression of a gene encoding the last third of MP (amino acids 187–264) in both types of plants did not. This suggests a major role for the N-terminal two-thirds of MP in the interaction with both the Tm-2 and Tm-2^2 proteins (Weber *et al.*, 2004). However, both with *Tm-2* and *Tm-2*^{2}, resistance observations were made that imply a role of the C-terminal third of MP in the interaction. In *Tm-2*-containing plants the induction of the necrotic response by MP with a deleted C-terminus was significantly delayed in comparison with the response elicited by the full-length protein. In addition, in *Tm-2*^{2}-containing plants, fusion of β-glucuronidase to the C-terminus of MP resulted in an absence of the necrotic response (Weber *et al.*, 2004).

Now, the observation can be added that the difference between the Tm-2 and Tm-2^2 R proteins is only four amino acids. This suggests that the interaction between the Tm-2 and Tm-2^2 proteins and MP or the MP/virulence target complex could be highly similar.

These observations can be reconciled with each other if one considers the proposed topological model of the MP of TMV, which suggests this protein is an integral membrane protein (Brill *et al.*, 2000). If one adapts the model of Brill *et al.* (2000) for the MP of ToMV it becomes clear that all changes in the *Tm-2*- and *Tm-2*^{2}-overcoming strains, except one, are located in the putative cytoplasmic domain of the MP. Moreover, it is very well possible that, due to the folding of MP, the domains in which the respective mutations are necessary to overcome either *Tm-2* or the *Tm-2*^{2} resistance interact or are close together. It is, therefore, possible that the two positionally differing sets of mutations are able to affect two highly similar interactions between the two R proteins and MP (or the complex involving MP). Close proximity or interaction of the two domains could also explain the absence of virus strains able to overcome both the *Tm-2* and *Tm-2*^{2} resistance by

harbouring both sets of mutations (Fraser *et al.*, 1989). If both the set of changes for breaking *Tm-2* and the set for breaking *Tm-2²* affect the same area of MP, the combined presence of both sets could then have a fatal impact on the function of MP.

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